Genome-CRISP™ CRISPR-Cas9 Products and Services

User Manual

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USER MANUAL

Genome-CRISP™ CRISPR-Cas9 Products and Services

I.Introduction
II.Related Services
III.Overview of Genome Editing Using CRISPR-Cas9
IV.Critical Steps
V.References
VI.Appendix
    A. Clone Products and Services
    B. Other Products and Services
VII.Licensing and Warranty Statement

I. Introduction

Background of CRISPR-Cas9 system

The clustered, regularly interspaced, short palindromic repeats (CRISPR)-associated protein (Cas) systems are adaptive mechanisms evolved by bacteria and archaea to destroy invading viruses and plasmids. Recently, efficient genome editing by the CRISPR-Cas9 system has been shown in multiple organisms, including zebrafish, mice, rats, C. elegans, plants, and bacteria. Several groups have demonstrated that, compared with zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), CRISPR-Cas9 – mediated gene targeting has similar or greater genome editing efficiencies in multiple systems.

Efficient and flexible targeting

In the CRISPR-Cas9 system, the complex of a CRISPR RNA (crRNA) annealed to a trans-activating crRNA (tracrRNA) is sufficient to guide the Cas9 endonuclease to a specific genomic sequence to execute gene-editing functions, such as gene knockout, knockin (with donor plasmid), modification and more. This system can be simplified by fusing the crRNA and tracrRNA sequences to produce a synthetic chimeric single-guided RNA (sgRNA). The selected target consists of a 20bp DNA sequence in the sgRNA, followed by a trinucleotide (5'-NGG-3') protospacer adjacent motif (PAM) which is recognized by Cas9 and is essential for gene editing functions. The sgRNA hybridizes to the strand complementary to the PAM site. Subsequently, the Cas9 nuclease cleaves both strands of the DNA (Figure 1).

This RNA-guided DNA recognition mechanism of CRISPR-Cas9 provides a simple but powerful tool for precise genome engineering. One of the most important advantages of CRISPR-Cas9 systems is that the Cas9 protein can be guided by individual sgRNAs to modify multiple genomic target loci simultaneously.

Advantages

- RNA-guided sequence-specific genome editing
- Simple and fast design process. No need to reengineer the nuclease for each new target
- Efficient RNA-guided DNA recognition regardless of the methylation state of the target site
- Flexible and robust multiplexing – edit multiple genome sites simultaneously

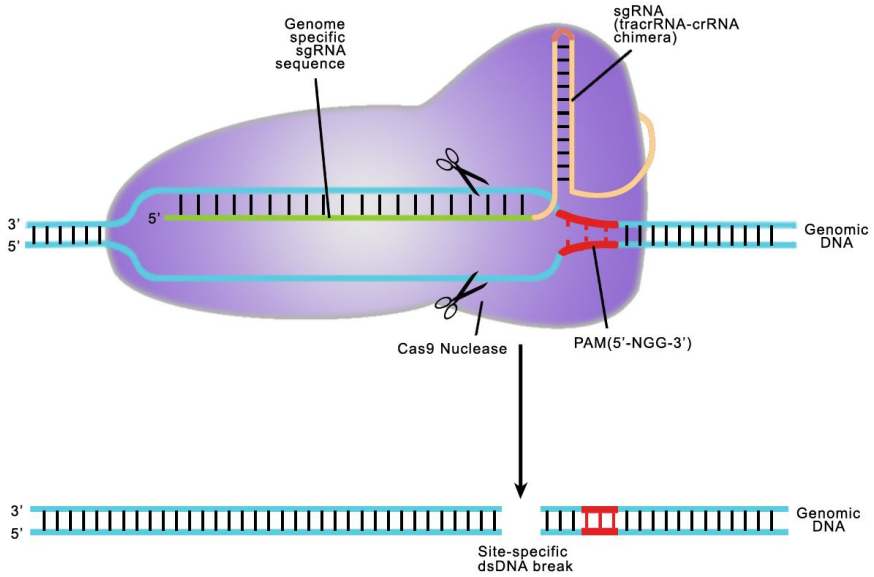


Figure 1. Illustration of CRISPR/Cas9-mediated genome editing

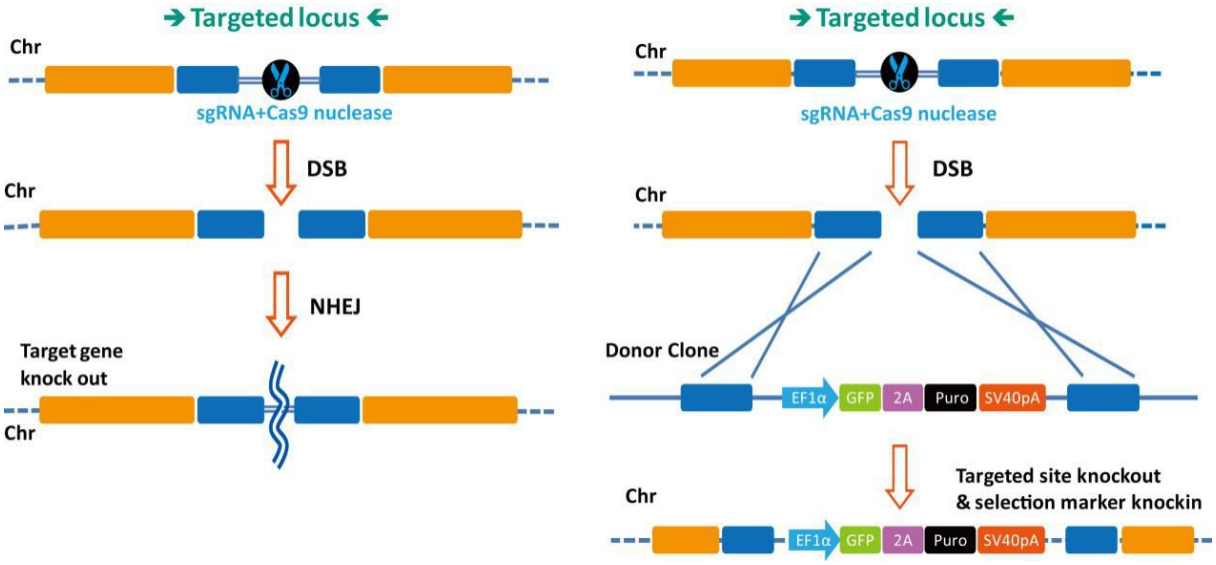
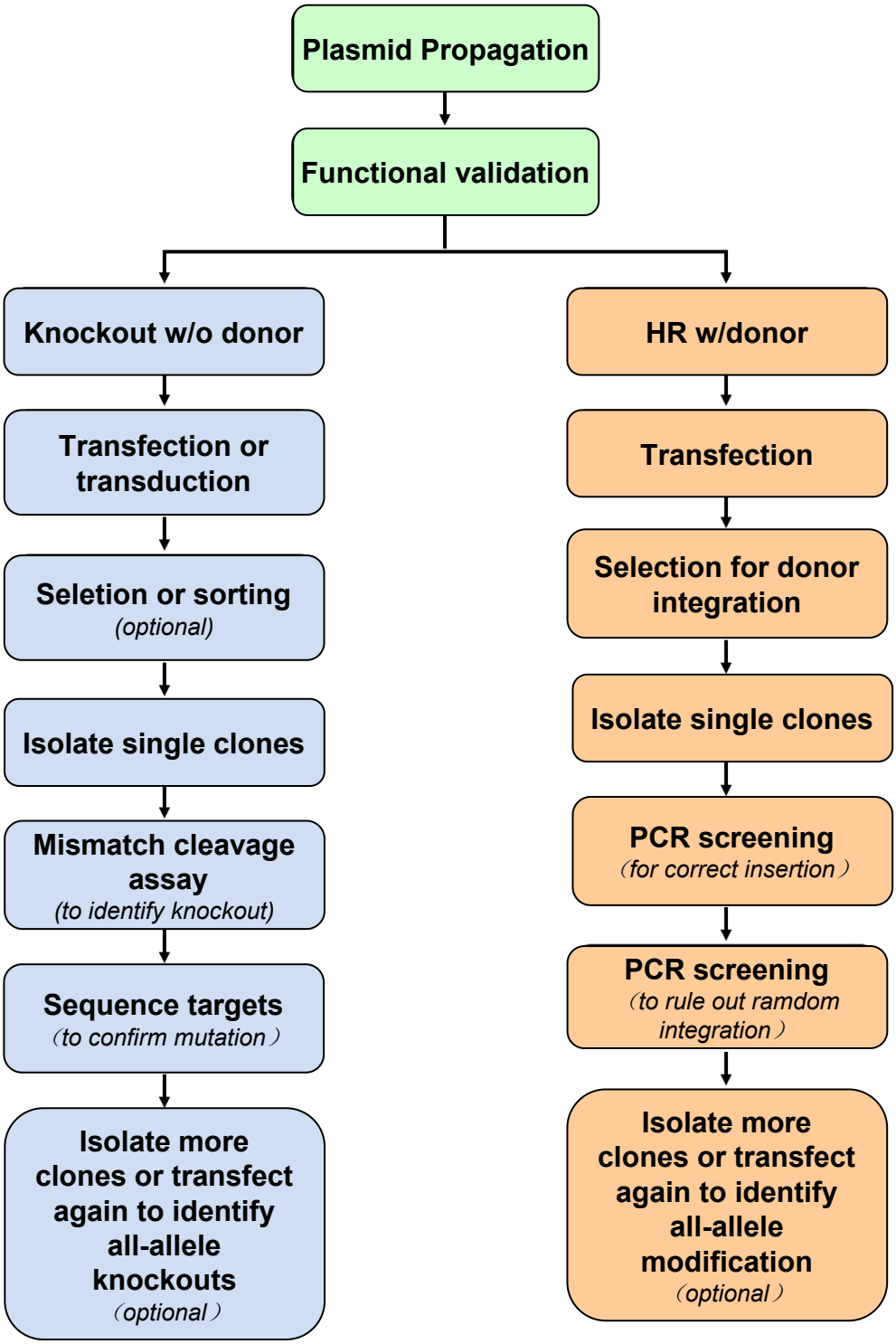


Figure 4. sgRNA-guided gene engineering. Left: DSB created by sgRNA-guided Cas9 nuclease is repaired by NHEJ. **Right:** DSB is repaired by the insertion of GOI & selection markers (or other genetic elements) from a donor plasmid via HR.

II. Related Product and Services

Services		Description
Validation	Mismatch cleavage assay	Chromosomal-level functional validation. Detects the presence of indels created by CRISPR-Cas9 mediated NHEJ repair at the specific target site of the chromosome.
Donor clone services (see appendix)	Donor clone design and construction	Customized plasmids designed to specifically transfer your gene of interest, selection marker or other genetic elements into targeted sites through homologous recombination (HR) induced by our CRISPR-Cas9. We offer various donor vector choices with different selection markers and genetic elements built in for your experiment purpose.
Stable cell line services (see appendix)	Monoclonal colony	Monoclonal stable cell line with CRISPR-Cas9-mediated genome modifications.
	Cell bank	Creation of a cell bank of monoclonal stable cell line with CRISPR-Cas9-mediated genome modifications.
Transgenic mouse services	Transgenic mouse	Transgenic mice with CRISPR-Cas9-mediated genome modifications.

III. Overview of Genome Editing Using CRISPR-Cas9


Plasmid Propagation
Functional validation
Knockout w/o donor
Transfection or transduction
Seletion or sorting (optional)
Isolate single clones
Mismatch cleavage assay (to identify knockout)
Sequence targets (to confirm mutation)
Isolate more clones or transfect again to identify all-allele knockouts (optional)
HR w/donor
Transfection
Selection for donor integration
Isolate single clones
PCR screening (for correct insertion)
PCR screening (to rule out ramdom integration)
Isolate more clones or transfect again to identify all-allele modification (optional)

IV.Critical Steps

A. Plasmid propagation

We recommend propagating the plasmids provided before your gene targeting experiment. Plasmids can be transformed using standard conditions suitable in any RecA- and EndA- E.coli competent cells.

For transformation of CRISPR-Cas9 product plasmids, we suggest plating 50-200µl of transformed cells on fresh LB-Ampicillin plates (50µg/ml). Incubate the plates at 37°C overnight. Inoculate colonies from the transformation and grow them at 37°C overnight in ~200ml of LB media containing 50µg/ml Ampicillin. Use an endotoxin-free plasmid DNA maxiprep kit to extract plasmid DNA after overnight growth.

To confirm the integrity of the amplified plasmids, we recommend restriction digestion analysis or direct sequencing.

B. CRISPR chromosomal validation

CRISPR-modified DNA will have a few bases of sequence inserted or deleted near the Cas9 cut site due to NHEJ exonuclease activity. We recommend using the GeneCopoeia's IndelCheck™ CRISPR/TALEN insertion or deletion detection system (ICPE-050, ICPE-200). Alternatives include the Cel1, mung bean and S1 nucleases.

The T7 endonuclease I assay is carried out according to the user manual of the IndelCheck™ detection system. We provide brief details here.

1. 24 or 48 hr post-transfection, collect cells for lysis (recommended) or for genomic DNA extraction.

2. Use target PCR kit (TPCR-050, TPCR-200) to amplify the region surrounding the sgRNA target site.

3. Check the PCR result by running 5 µL of PCR product on a 2% agarose gel. For all templates, it is important to make sure that there is only a single band corresponding to the intended product for the primer pair. The size of this band should be the same as calculated from the distance between the two primer annealing sites in the genome.

CRITICAL STEP: If multiple amplicons are generated from the PCR, redesign the primers and reoptimize the PCR conditions to avoid off-target amplification. In difficult cases in which a single-band product cannot be achieved, it is acceptable to gel-extract the correct-length band before proceeding with heteroduplex reannealing and Surveyor nuclease digestion.

4. DNA heteroduplex formation. At this point, the amplified PCR product includes a mixture of both CRISPR-modified and unmodified genomic DNA. Place 300 ng of the PCR product in a thermocycler tube and perform the cross-hybridization.

5. T7 endonuclease I assay. Treat the cross-hybridized homo- and heteroduplexes with T7 endonuclease I assay kit to determine CRISPR cleavage efficiency.

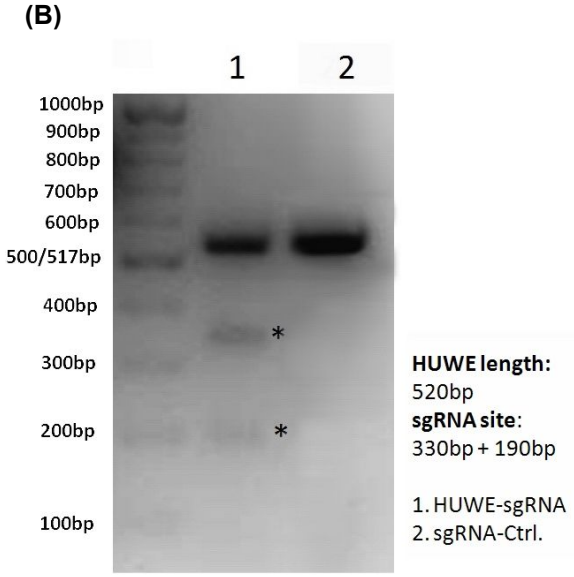


Figure 3. Mutagenesis of the human HUWE gene using CRISPR-Cas9.

(A) HUWEI sgRNA design. Expected bands of T7 endonuclease I (T7 ENI) digestion are 330bp + 190bp;

(B) The HEK293T cells were transfected with HUWE I - sgRNA / Cas9 clone (Lane 1) and scrambled-sgRNA / Cas9 control clone (Lane 2) in a 6-well plate. The cells were harvested 40h post transfection. The genomic DNA was extracted and PCR amplified using HUWE-specific PCR primer. The PCR products were purified by gel purification. 8uL purified PCR products were mixed with buffer, denatured and reannealed, and then digested with T7 ENI at 37°C for 60mins. The expected HUWE PCR product size is 520bp and the expected T7 ENI digestion product sizes are 330bp and 190bp.

C. Co-transfection (or transfection) into target cells

1. Plate ~100,000 to 300,000 cells/well in a 6-well plate following the recommended conditions for the cell type(s) being transfected. Scale up and down the culture if needed. On the day before transfection, trypsinize and count the cells. The number of cells plated in each well should be determined so that they are 70-80% confluent at the time of transfection.

2. The next day, prepare transfection complexes using suitable transfection reagents according to the manufacturer's instructions. Leave the transfection complexes on the cells to react for >6 hours.

Tech Notes:

1) Since transfection efficiencies vary across different cell lines, we recommend optimizing the input for best results.
2) For optimal results, we recommend complexing DNA with transfection reagent in serum- and antibiotic-free media and cells growing in complete media (e.g. DMEM/F12+10% FBS w/o antibiotics).
3) For hard-to-transfect cells (e.g. primary, stem, hematopoietic), it may be advisable to utilize a non-passive transfection method. Please follow recommended guidelines provided by the manufacturer for the specific cell type(s) being transfected.

3. 24 hours post-transfection, remove transfection media and split the cells 1:10 and 1:20 in complete growth media w/antibiotics. Plate cells into 6-well plates and save a set of plate(s) for characterization. Allow cells to recover for 24 hours.

4. Begin antibiotic selection 48 hours post-transfection. We recommend optimizing concentration of antibiotic for best results.

5. For HR-based knockin,. another round of antibiotic selection to enrich clones with donor integration is recommended.

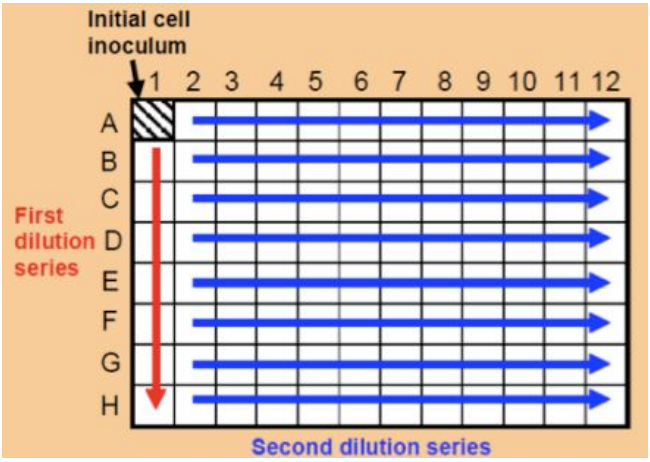
Tech Note:

Establishing a kill-curve on untransfected cells can determine the effective working antibiotic concentration for a target cell line. The concentration of antibiotic that kills >90% of cells after 48hours of selection is the correct dose for the cells being selected.

D. Clonal isolation of cell lines

Serial dilution is widely used to isolate single clones with desired modifications, followed by an expansion period to establish a new clonal cell line. Like most clonal isolation methods, there is no guarantee that the colonies arose from single cells. A second round is advised to increase the likelihood of clonal isolation. Also, it is worth noting that cell types can vary substantially in their responses to single-cell isolation, so literature specific to the cell type of interest should be consulted.

1. Fill each well of a sterile 96-well plate with 100 µL of medium except for well A1, which should remain empty.



2. Add 200µL cell suspension to well A1. Mix 100 µL from A1 with the medium in well B1. Avoid bubbles. Continue this 1:2 dilution through column 1. Add 100 µL of medium back to column 1 so that wells A1 through H1 contain 200 µL.

3. Mix cells and transfer 100 μL of cells from column 1 into column 2. Mix by gently pipetting. Avoid bubbles. Repeat these 1:2 dilutions through the entire plate. Bring the final volume to 200 μL by adding 100 μL of medium to all but the last column of wells.

4. Incubate plates undisturbed at 37℃.

5. Cells will be observable via microscopy over 3 days and be ready to score in 5-8 days, depending on the growth rate of cells. Mark each well on the cover of the plate indicating which well contains a single colony. These colonies can later be subcultured from the well into larger vessels.

Tech Note:

1) Adding 4000 cells in well A1 (2×104 cells/mL) is a good starting concentration. Increase the concentration for more difficult to grow cell lines.
2) If the reporter gene is fluorescent, determine which of these colonies express it. If the reporter gene is not observable you will have to wait until later in the culture process.
3) Label each well with a single colony using a unique identification number and record this number on the plate and in your notebook.

E. Validation of CRISPR-Cas9 modified and HR recombinant cells

1. To confirm donor vector integration specifically at a target site, junction-PCR can be performed using PCR primer pairs that flank the 5' homology arm and 3' homology arm.

2. Protocol for Junction-PCR

1) Primers should be diluted to 10μM before use. Validation of either the 5' or 3' homology arms for donor integration is usually sufficient; however, both arms can be done for additional confirmation.

2) Protocol details for junction-PCR assay:
   a) Isolate genomic DNA from positive control cells or test sample cells using a suitable genomic DNA miniprep kit. Please follow the protocol recommended by the manufacturer.
   b) Perform junction-PCR (PCR reaction below)

Reagent	CRISPR-Cas9 cut+ positive control donor	Positive control donor only
Genomic DNA(60~100ng/μl)	1μL	1μL
10μM 5' (or 3') PCR Primer Mix	1μL	1μL
5×UltraPFTM Buffer (Mg2+ free)	5μL	5μL
10 mM dNTPs	0.5μL	0.5μL
20mM MgSO4	2.5μL	2.5μL
UltraPF(5U/μl)	0.25μL	0.25μL
PCR-grade distilled water	14.75μL	14.75μL
Total	**25μL**	**25μL**

98°C, 5min

98°C, 20sec

55°C, 30sec ┐ 35 cycles

72°C, 1min ┘

72°C, 7min

Hold at 4~16°C

Run the PCR reaction out on the 1% Agarose/EtBr gel in 1X TAE buffer to confirm the junction-PCR result.

Sample results for 5' and 3' junction-PCR assay depend on design.

Tech Note:

1) The 3' junction PCR band and 5' junction PCR band may differ in brightness because the amplification efficiency may be different due to the nature of the chromosomal structure, modification and sequence around that region.
2) One positive in junction PCR is sufficient to confirm the integration.
3) It is possible that random integration can coexist with site-specific integration. Negative selection can be used to detect coexisting random integration.

V. References

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3. Hale CR, Zhao P, Olson S, et al. (November 2009). "RNA-Guided RNA Cleavage by a CRISPR RNA-Cas Protein Complex". Cell 139 (5): 945–56.

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5. Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., and Charpentie E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptiv bacterial immunity. Science 337, 816–821.

6. Jiang, W., Bikard, D., Cox, D., Zhang, F., and Marraffini, L.A. (2013). RNA-guided editing of bacterial genomes using CRISPR-Cas systems. Nat.Biotechnol. 31, 233–239.

7. Hsu, P.D., Scott, D.A.,Weinstein, J.A., Ran, F.A., Konermann, S., Agarwala, V.,Li, Y., Fine, E.J., Wu, X., Shalem, O., et al. (2013). DNA targeting specificity of RNA-guided Cas9 nucleases. Nat. Biotechnol. Published online July 21, 2013.

VI. Appendix

A. Clone Products and Services

Genome-CRISP™ Cas9 Nuclease and D10A Nickase Expression Clones

A Cas9 clone expresses human codon-optimized Cas9 nuclease from Streptococcus pyogenes. A Cas9 nickase clone expresses a mutant of Cas9 carrying an aspartate-to-alanine substitution at amino acid position 10 (D10A).

In concert with a single guide RNA (sgRNA), the Cas9 nuclease creates a site-specific double-strand break (DSB) at a 20 nucleotide target site that is immediately followed by an N-G-G protospacer adjacent motif (PAM). DSBs are repaired by either nonhomologous end joining (NHEJ), which is error-prone and leads to frameshift mutations, or by homologous recombination (HR) in the presence of a repair template. The ability of CRISPR to greatly stimulate DSBs has been exploited to knock genes out, or create knockins of point mutations, reporters, or other modifications in many experimental systems.

The Cas9 D10A nickase uses the same recognition system as the wild type Cas9, except that it can create only single strand “nicks”, which are not usually repaired by NHEJ or HR. However, when two correctly oriented sgRNAs are targeted to opposite strands flanking a desired site, a DSB is generated and then repaired efficiently by NHEJ or HR. This double nicking strategy is often used for applications demanding lower off-target modification.

Catalog#	Product	Promoter	Reporter gene / Selection marker
CP-C9NU-01	Cas9 nuclease expression clone	CMV	mCherry / Neomycin
CP-LvC9NU-01	Cas9 nuclease lentiviral expression clone	CMV	Neomycin
CP-LvC9NU-02	Cas9 nuclease lentiviral expression clone	CMV	eGFP / Neomycin
CP-C9NI-01	Cas9 D10A nickase expression clone	CBh	N/A
CP-C9NI-02	Cas9 D10A nickase expression clone	CMV	mCherry / Neomycin

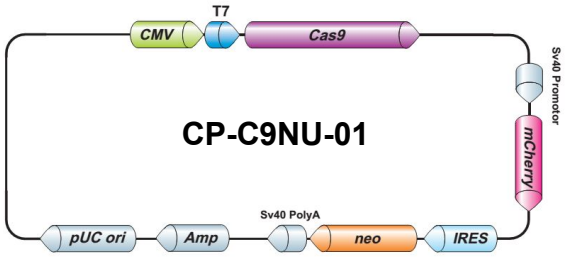


Figure 4. Map of Cas9 nuclease expression clone

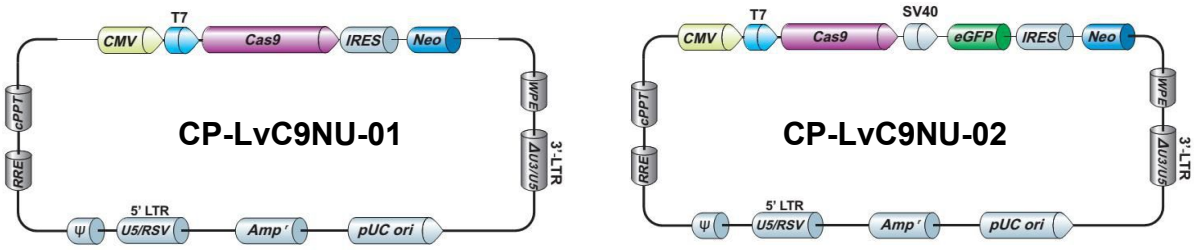


Figure 5: Maps of Cas9 nuclease lentiviral expression clone

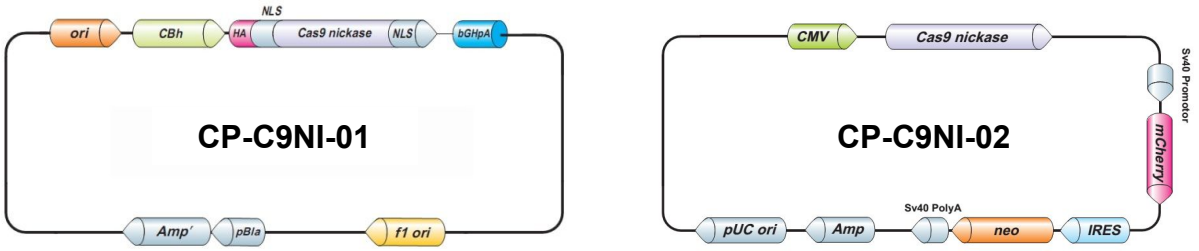


Figure 6: Maps of Cas9 Nickase Expression Clone

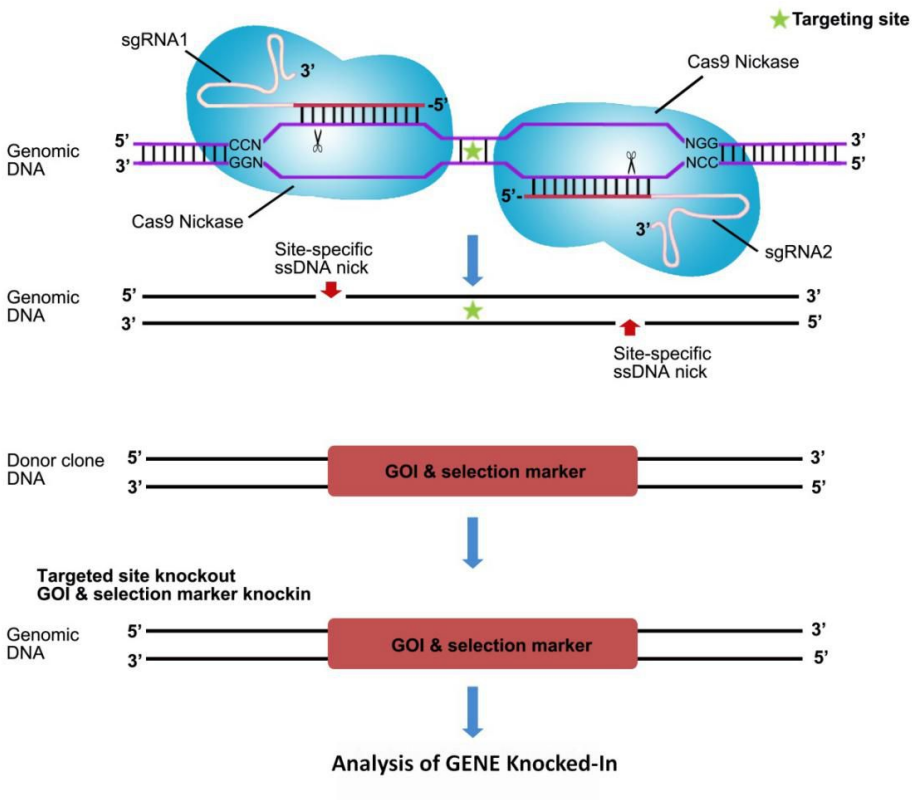


Figure 7. Illustration of the "double nicking" strategy using the Cas9 D10 nickase mutant.

Genome-CRISP™ sgRNA Design and Cloning Services

GeneCopoeia offers single-guide RNA (sgRNA) design and cloning services for the customer's target gene of interest. sgRNA clones express a single-stranded chimeric sgRNA, consisting of crRNA and tracrRNA. In the presence of the co-transfected Cas9 endonuclease, sgRNA can recognize the targeted DNA sequence and guide Cas9 nuclease to create DSBs and facilitate genome editing function for gene knockout, knockin, mutagenesis, and more. Multiple sgRNA clones can be constructed and co-transfected with one Cas9 clone to enable simultaneous editing of several sites within the genome, offering greater efficiency and flexibility for the experiment design.

Vector	Promoter	sgRNA and Cas9	Selection Marker/ Reporter Gene
pCRISPR-SG01	U6	sgRNA only*	Hygromycin
pCRISPR-LvSG03	U6	sgRNA only*	Puromycin/mCherry
pCRISPR-CG01	U6	sgRNA and CMV-driven Cas9 (wild type) in the same all-in-one vector	Neomycin / mCherry
pCRISPR-CG02	U6	sgRNA and CBh-driven Cas9 (wild type) in the same all-in-one vector	N/A

*Compatile Cas9 Nuclease (Cat.No. CP-C9NU-01, CP-LvC9NU-01, CP-LvC9NU-02) or Cas9 D10A Nickase (Cat.No.CP-C9NI-01, Cat.No.CP-C9NI-02) are available as separate expression clones.

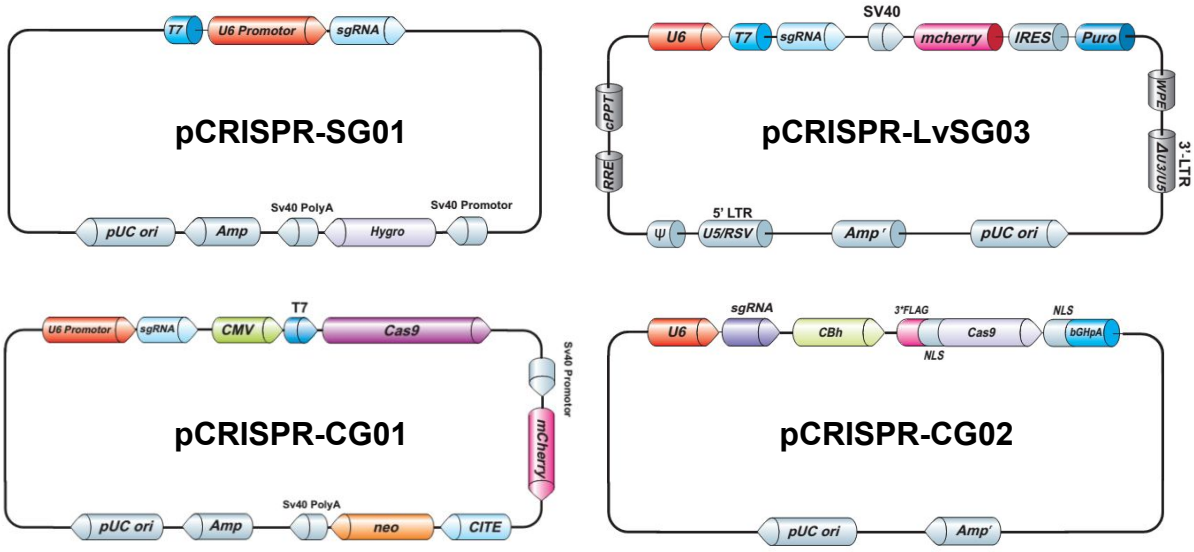


Figure 7. Maps of sgRNA clones

A. Other Products and Services

Donor services

GeneCopoeia offers customized donor clone design and construction services. Donor clones are customized plasmids designed to specifically transfer your gene of interest, selection marker or other genetic elements into a target site via HR-mediated repair of DSBs induced by site-specific genome editing tools. Donor vectors are available with several options for selection markers and genetic elements to meet your experimental needs.

Donor vector types

Vector	Promoter	Reporter Gene	Selection Marker	LoxP Site
pDonor-D01	EFa1	copGFP	Puromycin	N/A
pDonor-D02	CMV	copGFP	Neomycin	N/A
pDonor-D03	CMV	N/A	Neomycin	N/A
pDonor-D04	CMV	N/A	Puromycin	N/A
pDonor-D05	EFa1	N/A	Neomycin	N/A
pDonor-D07	EFa1	copGFP	Puromycin/TK	Loxp
pDonor-D08	CMV	copGFP	Neomycin/TK	Loxp
pDonor-D09	EFa1	N/A	Puromycin/TK	Loxp
pDonor-D10	CMV	N/A	Neomycin/TK	Loxp
pDonor-D11	PGK	copGFP	Puromycin/TK	Loxp
pDonor-D12	EF1a	copGFP	Hygromycin/TK	Loxp
pDonor-D13	PGK	copGFP	Neomycin/TK	Loxp
pDonor-D14	PGK	N/A	Puromycin/TK	Loxp

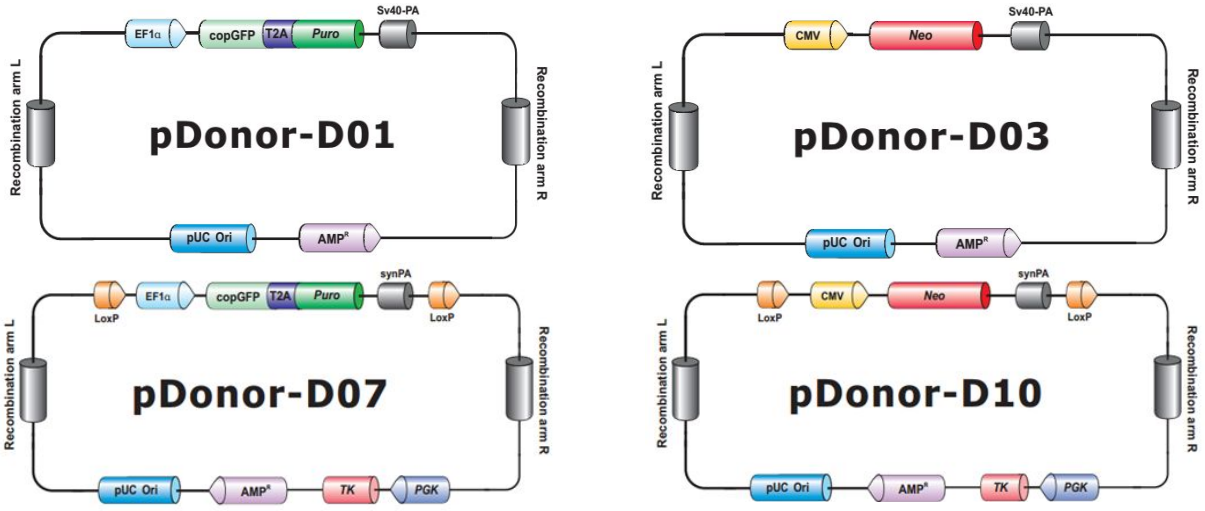
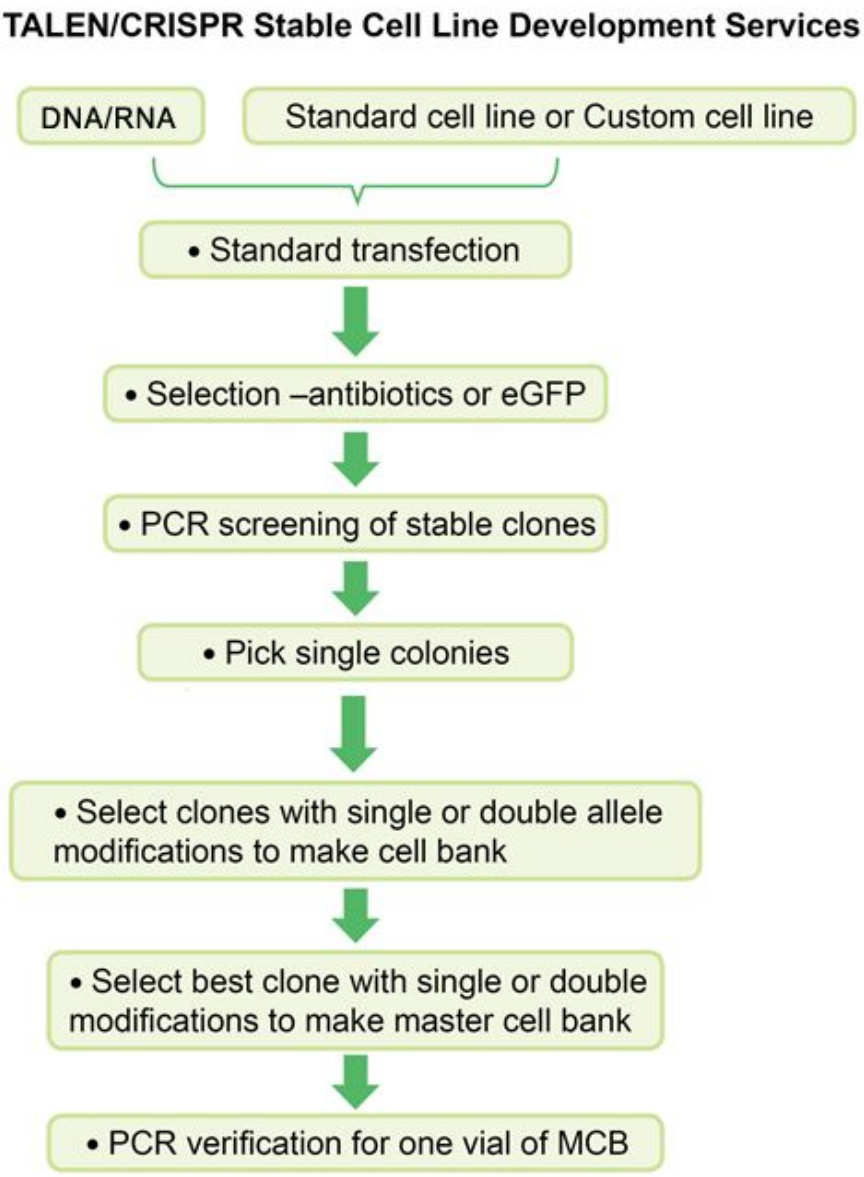


Figure 8. Maps of some of the donor vectors

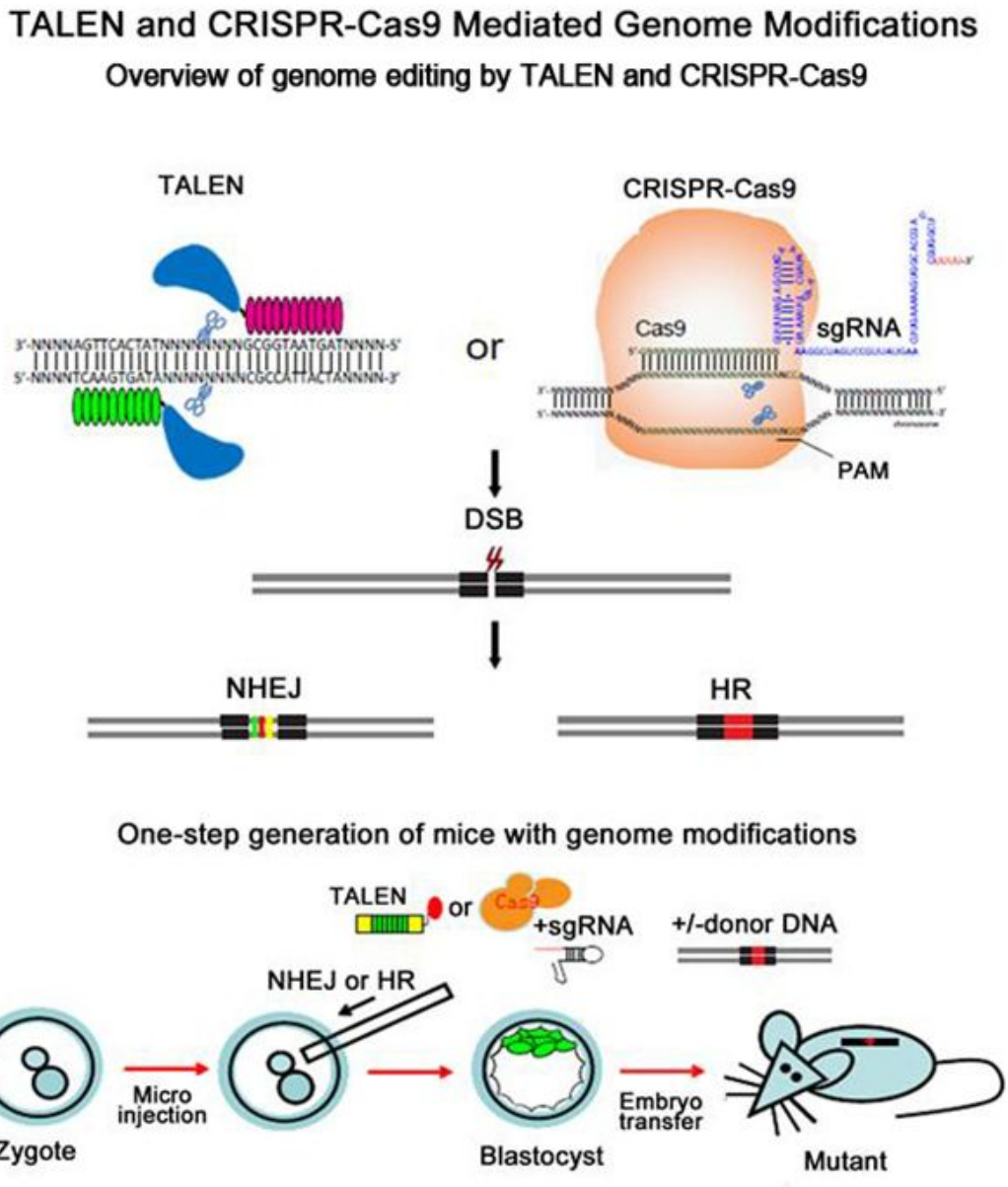
Stable cell line services

GeneCopoeia offers monoclonal stable cell line service with customized TALEN-mediated genome modifications. Cell banking service is also available.


TALEN/CRISPR Stable Cell Line Development Services
DNA/RNA
Standard cell line or Custom cell line
• Standard transfection
• Selection –antibiotics or eGFP
• PCR screening of stable clones
• Pick single colonies
• Select clones with single or double allele modifications to make cell bank
• Select best clone with single or double modifications to make master cell bank
• PCR verification for one vial of MCB

Transgenic mouse services

Transgenic mouse models are now considered ideal tools to delineate the molecular mechanisms of the gene products and their interactions with one another that influence all cellular processes that form the basis of physiological systems. . GeneCopoeia offers transgenic mice with customized TALEN- or CRISPR-Cas9-mediated genome modifications.



VII. Licensing and Warranty Statement

Limited Use License

Following terms and conditions apply to use of the Genome-TALERTM Human AAVS1 Safe Harbor Gene Targeting Kit (the Product). If the terms and conditions are not acceptable, the Product in its entirety must be returned to GeneCopoeia within 5 calendar days. A limited End-User license is granted to the purchaser of the Product. The Product shall be used by the purchaser for internal research purposes only. The Product is expressly not designed, intended, or warranted for use in humans or for therapeutic or diagnostic use. The Product must not be resold, repackaged or modified for resale, or used to manufacture commercial products or deliver information obtained in service without prior written consent from GeneCopoeia. This Product should be used in accordance with the NIH guidelines developed for recombinant DNA and genetic research. Use of any part of the Product constitutes acceptance of the above terms.

Limited Warranty

GeneCopoeia warrants that the Product meets the specifications described in the accompanying Product Datasheet. If it is proven to the satisfaction of GeneCopoeia that the Product fails to meet these specifications, GeneCopoeia will replace the Product. In the event a replacement cannot be provided, GeneCopoeia will provide the purchaser with a refund. This limited warranty shall not extend to anyone other than the original purchaser of the Product. Notice of nonconforming products must be made to GeneCopoeia within 30 days of receipt of the Product. GeneCopoeia's liability is expressly limited to replacement of Product or a refund limited to the actual purchase price. GeneCopoeia's liability does not extend to any damages arising from use or improper use of the Product, or losses associated with the use of additional materials or reagents. This limited warranty is the sole and exclusive warranty. GeneCopoeia does not provide any other warranties of any kind, expressed or implied, including the merchantability or fitness of the Product for a particular purpose.

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